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Citation for published version:

Batrakou, DG, Kerr, ARW & Schirmer, EC 2009, 'Comparative proteomic analyses of the nuclear envelope and pore complex suggests a wide range of heretofore unexpected functions', *Journal of proteomics*, vol. 72, no. 1, pp. 56-70. <https://doi.org/10.1016/j.jprot.2008.09.004>

Digital Object Identifier (DOI):

[10.1016/j.jprot.2008.09.004](https://doi.org/10.1016/j.jprot.2008.09.004)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of proteomics

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**Comparative Proteomic Analyses of the Nuclear Envelope and Pore
Complex Suggests a Wide Range of Heretofore Unexpected Functions**

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Abstract

Since the discovery of several inherited diseases linked to the nuclear envelope the number of functions ascribed to this subcellular organelle has skyrocketed. However the molecular pathways underlying these functions are not clear in most cases, perhaps because of missing components. Several recent proteomic analyses of the nuclear envelope and nuclear pore complex proteomes have yielded not only enough missing components to potentially elucidate these pathways, but suggest an exponentially greater number of functions at the nuclear periphery than ever imagined. Many of these functions appear to derive from recapitulation of pathways utilized at the plasma membrane and from other membrane systems. Additionally, many proteins identified in the comparative nuclear envelope studies have sequence characteristics suggesting that they might also contribute to nuclear pore complex functions. In particular, the striking enrichment for proteins in the nuclear envelope fractions that carry phenylalanine-glycine (FG) repeats may be significant for the mechanism of nuclear transport. In retrospect, these findings are only surprising in context of the notion held for many years that the nuclear envelope was only a barrier protecting the genome. In fact, it is arguably the most complex membrane organelle in the cell.

Keywords: nuclear envelope; nuclear pore complex (NPC); tissue variation; phenylalanine-glycine (FG) repeats; nuclear transport; comparative proteomics.

1. Introduction

The structure of the nuclear envelope (NE) is complex (for reviews see [1-4]). It is a double membrane system continuous with the endoplasmic reticulum (ER) that consists of three connected but distinguishable membrane domains: the outer, inner and pore membranes (Fig. 1A). The outer nuclear membrane (ONM) is studded with ribosomes [5,6] and contains many ER proteins in addition to having a set of unique proteins, some of which appear to be involved in tethering the nucleus to cytoplasmic filament systems [7-12]. Some of these proteins in turn connect across the lumen of the nuclear envelope to the inner nuclear membrane (INM), which contains its own unique set of proteins (Fig. 1B; reviewed in [3,13]). Many of these have been shown to bind both to the intermediate filament lamin polymer [14-19] and chromatin (reviewed in [20]). Nuclear pore complexes (NPCs) are inserted at the pore membrane (PoM) that connects the ONM and INM (Fig. 1A; reviewed in [4]). The NPCs regulate directional transport of proteins and mRNA between the nucleus and cytoplasm that exceed the measured maximum free diffusion limit of ~40-60 kDa [21] and can accommodate very large molecules or complexes that have been experimentally tested up to 39 nm diameter [22] (to put this in perspective the longest dimension of an assembled ribosome is ~25 nm). To accommodate such large substrates, the NPCs are necessarily large, on the order of 44 to 125 MDa. These large complexes are tethered to the membrane by at least three unique transmembrane proteins [23-28] that also contribute to a ring component of the NPC in the NE lumen [29-31]. The lumen of the nuclear envelope is largely unexplored territory, but is likely to have its own unique functions.

NE proteins have now been shown to influence a wide range of functions, although it is unclear whether their effects are direct or indirect. These functions include nuclear morphology and stability [32-36], nuclear anchoring/ migration within the cell [8-10], signaling cascades [37-40], and support of DNA replication [41-43], transcription [44-46], and RNA splicing [47]. Consistent with the notion that the nuclear lamina carries out or facilitates a diverse range of basic cellular functions, over a dozen inherited diseases and syndromes are linked to lamins and certain associated NE proteins. NPC proteins have also been linked to disease. These nuclear envelope diseases include muscular

dystrophies, lipodystrophies, neuropathy, cardiomyopathies, dermatopathy, bone disorders, and premature aging syndromes (reviewed in [48-51]). The proteins thus far linked to disease are lamins A/C, B1, and B2, emerin, LBR, LAP2, MAN1, Syne/Nesprin-1, FACE-1/ZMPSTE24, torsin A, and the NPC proteins Aladin, and Nup62. The favored hypotheses to explain how these proteins can yield so many different diseases are: 1) reduced resistance to mechanical stress, 2) disruption of gene regulation, 3) alterations in cell cycle and signaling pathways. However, none of these hypotheses can fully explain by themselves how mutations in the same widely expressed proteins can yield different diseases that each have distinctive tissue pathologies. Correspondingly, it is hard to imagine the many functions ascribed to lamins and associated proteins being due to diverse enzymatic activities encoded within the same proteins. The resolution in both cases likely involves additional partner proteins that provide these functions and have yet to be identified, hence the need for proteomic analysis.

The combination of its inclusion of cytoskeletal elements, the lamin polymer, integral membrane proteins, NPCs, a membrane and its luminal content that is continuous with the ER together with the many associations with chromatin proteins, transport receptors and cargos, and indirectly attached peripheral components of the cytoskeleton give the NE a wide range of biochemical properties. This has the consequence that any biochemical fractionation methodology will necessarily remove some true NE components and bring along some contaminants. As the ONM is continuous with the ER and connected to cytoplasmic filament systems [9,52] these structures are difficult to separate. INM proteins bind chromatin (reviewed in [20]) and in yeast the NPCs are connected to nucleoplasmic filaments [53,54] that in turn connect to telomeres [55,56]. All of these connections on both cytoplasmic and nucleoplasmic faces of the NE and NPC further compound difficulties in their purification.

2. The Nuclear Pore Proteome

2.1 Pre-proteomics studies

As one of the largest macromolecular complexes in biology it is not surprising that most of the original characterization of the NPC was through the electron microscope. This work determined that the *Xenopus* NPC had a diameter of ~120 nm with eight-fold radial symmetry perpendicular to the membrane and a predicted mass of roughly 125 MDa [29,31,57] while the yeast NPC was somewhat smaller at 55-72 MDa [58,59]. Thus the NPC could be made up of a very large number of distinct proteins. Determining its composition was therefore not trivial even in the eras of genomics and proteomics. As the average mammalian nucleus has 2-3,000 NPCs [60] identification of NPC component proteins by biochemical fractionation was initially attempted and was successful in some cases. For example the first individual NPC component identified was gp210 that was isolated from a rat liver NE fraction and used to make antibodies that labeled the NPCs by immunogold electron microscopy ([61]; note this was originally called gp190 and renamed after the gene was cloned 7 years later [28]). Many other NPC proteins were soon identified using similar approaches and the proteins were called nucleoporins or NUPs [62-64]. Many of these antibodies cross-reacted among mammals, *Xenopus* and yeast [65-67] thus facilitating cloning of NUPs. *Xenopus* oocytes were the best system for biochemical purification while yeast was the most genetically tractable, so that many of the first NUP sequences were from yeast (*e.g.* NUP1 [66], NSP1 [68]). Once the first NUPs were identified it was discovered that they tend to form subcomplexes with 3-5 proteins and this rapidly facilitated further NUP identifications. For example p62 was among the first NUPs cloned [69] due to its abundance and strong antigenicity and it was subsequently found to be part of a complex with p54 and p58 in mammalian cells [70,71]. In yeast Nup170p was found to be in a complex with Nup53p and Nup59p [72] and Nic96p was identified through its interaction with Nsp1p [73]. Many individual studies over nearly two decades had identified 26 core NPC components in yeast (Table 1) by the time that the first major proteomic studies were done on the NPC. However, absent knowing how many copies of each protein were in each NPC it was impossible to gauge how many more components still needed to be identified and it was generally thought that between 50 and 100 proteins would be required to account for the estimated 55 to 125 MDa mass predicted by electron microscopy.

2.2 The yeast NPC proteome

The first comprehensive determination of NPC composition was the product of collaboration between the Rout, Aitchison and Chait laboratories [74]. Critical to a successful proteomic study is the choice of experimental system and the purity of the fractions analyzed. This study used yeast (*Saccharomyces cerevisiae*) because it had the highest NPC:nuclear volume ratio of any organism tested [75]. A haploid yeast nucleus has 65-182 NPCs depending on the cell cycle stage [76]. Unlike mammalian cells yeast do not have a lamina, which has the advantage of less contamination from connections to such a promiscuous structure and the disadvantage of losing the added stability to NEs from the lamina during purification. Yeast cells were spheroplasted, lysed and nuclei were isolated on sucrose gradients. The chromatin was enzymatically digested and extracted with heparin to isolate NEs. NPCs were released from NEs with mild detergent followed by isolation through their partitioning on a continuous sucrose velocity gradient [58]. Three separate rounds of separation by HPLC and SDS-PAGE yielded 465 protein bands that were digested and analyzed by matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and an additional 177 protein bands were analyzed by MALDI-ion trap tandem mass spectrometry. A total of 174 protein identifications were made [74]. Among these were all previously characterized NUPs, many proteins with known functions at the NPC such as transport factors and chaperones, many other proteins with known functions elsewhere that were considered to be contaminants, and 34 previously uncharacterized ORFs.

All previously uncharacterized ORFs and several known NUPs were genomically tagged with a protein A epitope and tested in yeast for their localization at the NE by immunofluorescence and specifically at the NPC by immuno-electron microscopy. Those that fulfilled these criteria and also were significantly enriched in a NE fraction by Western blot were classified as core NPC components, a total of 30 proteins (two of which are products of the same gene). Thus the yeast proteomic analysis identified only 4 additional core NPC components on top of those previously identified (Table 1). An additional ten NPC-associated proteins that did not fulfill their stringent criteria for inclusion as core

components were classified among those identified, leaving 134 of the 174 proteins identified classified as contaminants by the authors.

2.3 The mammalian NPC proteome

Subsequently the Matunis laboratory determined the composition of the mammalian NPC [77]. They used rat liver because previous work had developed procedures for isolating extremely clean fractions of nuclear envelopes from this material [61,78-80]. These procedures take advantage of the relative softness of liver as a tissue to lyse cells without breaking their nuclei and also benefit from the stability conferred to the NE by the lamin polymer to allow several steps of douncing, floating contaminating membranes on sucrose cushions, chromatin digestion and salt washes without fragmenting NEs. For fear that some NPC components might be also removed by high salt treatments, they however replaced the salt with heparin and spermidine so that extraction would favor removal of just histones. They next took advantage of the biochemical characteristics of the lamin polymer and its binding partners that are highly resistant to extraction by high salt and detergent treatments and tested a variety of mild detergents for those that would solubilize NPC components while leaving lamina components insoluble. They found that all well-characterized NPC components could be extracted while leaving most of the lamina components in the pellet using a hypotonic solution containing a low concentration (0.3%) of the detergent Empigen BB. Similarly to the yeast study, this material was separated by HPLC prior to SDS-PAGE and analyzed using a combination of single step and tandem mass spectrometry. Previously uncharacterized proteins were tagged with GFP and tested for NPC colocalization using an antibody (mAb414) that recognizes O-linked glycosylation on several nucleoporins [62]. This study identified 23 proteins that were classified as core NUPs and 18 proteins classified as NPC-associated proteins (Table 1).

2.4 The revised NPC

The big surprise from both the yeast and mammalian studies was the relatively small number of proteins identified. Original estimates of protein content based on electron microscopy studies had predicted between 50 and 100 distinct protein components for the NPC [29,31,57]. The total number

of only ~30 can in part be explained by the observations that several components have well above average molecular weights and that many components are duplicated many times per NPC. Based on the eight-fold symmetry of the NPC core NUPs would be expected to be duplicated eight times, but the abundance of many components suggested that they are represented 16 and 32 times within a single NPC [74,77]. Furthermore, the yeast study excluded the Mlp (Tpr in mammals) proteins that make up much of a “nuclear basket” extending into the nucleus from the NPC that was observed in electron microscopy studies. Nonetheless, even taking into account these considerations, the total mass of the core NPC calculated from summing up the components identified in the proteomic analyses (44 MDa in yeast [74] and ~60 MDa in mammals [77]) was surprisingly lower than the mass predicted from electron microscopy studies (55-72 MDa in yeast [58,59] and 125 MDa in mammals [29,57]).

A potential explanation for this discrepancy may lie in the additional proteins that both studies identified, but excluded because they did not fulfill their very conservative definition for core NPC components. There are a great many NPC proteins that have only transient associations during the transport process. For example the Ran GTPase is a very abundant protein that is involved in release of transport substrates from NPC proteins in the nucleus (Fig. 2A). Importins and exportins are transport receptors that bind to the transport cargo and interact with “core” NPC components to facilitate transport of the cargo through the central channel of the NPC (Fig. 2B and C). These interactions presumably occur through repeat motifs containing phenylalanine and glycine (FG repeats) that appear on both NUPs and many transport receptors. Because their associations are transient neither Ran proteins nor transport receptors were considered as core NPC components, yet due to their high abundance these proteins were also identified in these studies and could moreover account for a significant portion of the mass difference between the calculations from the “core” NPC components and those measured from electron microscopy studies. Even with these, however, the mass difference of almost 2-fold for the mammalian NPC would likely indicate the existence of additional proteins not yet identified (either core components or transient NPC proteins) that were

extracted during the preparation of the core NPC fraction. Another possibility is that some of the proteins that were discounted because of other previously characterized cellular functions and localizations actually have separate functions at the NPC. For example the Sec13 proteins are known to function in vesicle formation in the ER [81], yet also were shown to function in nucleo-cytoplasmic transport [82]. Thus, additional NPC components might be identified if these studies were repeated using multiple and varied purification methodologies and if proteins identified with distinct known cellular functions were also tested for NPC function.

It is also worthy of note that the original 125 MDa mass estimate for the NPC of higher eukaryotes was actually principally determined from *Xenopus*, which was much more accessible to analysis than mammalian nuclei. Thus it is possible that the *Xenopus* NPC, not yet analyzed by proteomic approaches, is larger than either yeast or mammalian NPCs. Comparison of the yeast and mammalian NPCs would suggest that if this is the case the mass difference likely is accounted for mostly in more peripherally associated proteins because the differences between the yeast and mammalian proteins identified as core structural NPC components is smaller than it appears (Table 1). While several proteins did not have homologues, they nevertheless had analogues: although no clear sequence homology links yeast Pom152 to the mammalian Pom121, both are transmembrane proteins with similar functions. Likewise yeast Ndc1 had no clear mammalian homologue identifiable by genome mining and was not found in the mammalian NPC study, but the mammalian Ndc1 analogue was subsequently identified in a NE proteomic study ([26,83]; see below) giving direct support to the notion that differences in purification methodologies will also contribute to differences in identifications. The mammalian Nup50 appears to be an analogue of Nup2p in yeast, which was not considered in the yeast proteomic study to be part of the core because its association is too dynamic. Although we cannot tell if completely novel or as yet unidentified core components are part of the *Xenopus* NPC until it has been also analyzed by proteomics, nonetheless, blast searching yeast and mammalian nucleoporins against the *Xenopus laevis* and *Xenopus tropicalis* genomes indicates only

Nup133, PoM152, Gle1, Nup60, and PoM34 among the proteins listed in Table 1 do not have homologs in frogs.

Phylogenetic analysis of the sequences of these core NPC proteins brought another striking observation. The yeast NPC proteins could be separated into pairs that clearly diverged after a gene duplication event [84]. This is consistent with predictions based on the yeast genome sequence of a general genome duplication that was followed by asynchronous differentiation of the duplicated genes [85]. The gene duplications expanded the range of phenylalanine-glycine (FG) repeat motif NUPs and of beta barrel/ alpha solenoid fold NUPs. These latter are related to coatamer proteins and have been suggested to facilitate bending of the pore membrane during NE/ NPC assembly [84]. Thus, though at the sequence level not all NPC proteins are conserved, the central evolutionary mechanism underlying their development argues that they will be essentially conserved at the core.

3. The Nuclear Envelope Proteome

3.1 Approaches prior to proteomics

The abundance (~3 million copies per mammalian nucleus [60]) and biochemical properties of the intermediate filament lamins enabled their being the first NE proteins to be identified [78,86]. Following on this the first NE transmembrane proteins (NETs) identified were INM proteins that bound to lamins and so were resilient to extraction by procedures for isolating NEs that were based largely on the resistance of the supporting lamin polymer to high salt and detergents. The lamin B receptor (LBR) was so named because of its identification through its binding to lamin B1 [19] while LAP1 and LAP2 that also bind lamins were identified as major proteins in a NE/ lamina fraction that was used to generate monoclonal antibodies [15,87]. The methods of discovering the next round of NETs were surprisingly varied ranging from autoimmune antibodies that stained the NE in the case of MAN1 [88] to a 2-hybrid screen for partners of a kinase of the postsynaptic membrane in muscle that identified the Syne/Nesprin-1 proteins [7].

As different groups were using different organisms to identify NETs and the timing correlated with the beginning of large-scale genome sequencing projects, once the first several proteins had been identified, attempts were made to identify related proteins through sequence similarity. This type of analysis found the Syne/Nesprin-2 NE protein family from its sequence similarity to Syne/Nesprin-1 [7], but it did not identify the Nesprin-3 family that was subsequently identified by proteomics [12,83]. Moreover, the two first identified *Drosophila* NETs do not have mammalian homologs (Otefin [89] and YA [90]). Thus the only way to determine the components of the NE would be directly through proteomic analyses.

3.2 Strategies for NE proteome determination

The continuity between the ONM and the peripheral ER on one side and many connections to chromatin on the other require creative approaches to the identification of NE proteins by proteomics, especially after the high number of proteins defined as contaminants in the NPC proteomes (provided they are truly contaminants). Two studies that were relatively similar in purifications yet different in strategies yielded strikingly different results [83,91]. Both studies used equivalent procedures for isolating mammalian NEs as were used in the mammalian NPC study; however, the NEs were either extracted to enrich for proteins associated with the intermediate filament lamin polymer or to enrich for proteins embedded in the membranes.

The first study from the Otto laboratory generated three separate NE fractions: a chaotrope-insoluble fraction, a non-ionic detergent-insoluble fraction and a salt-insoluble fraction [91]. Extraction with chaotropes (4 M Urea, 200 mM sodium carbonate) solubilizes the lamin polymer and most protein-protein interactions, but has no effect on membranes so that proteins embedded in the membrane are protected and maintained in the membrane fraction. The extraction with detergent (1% Triton X-100) should remove all membranes and so only proteins tightly bound to the lamin polymer should remain. Finally extraction with high salt (1 M NaCl) should also leave the lamina intact, but wash away soluble proteins that are weakly associated with it. Each fraction was separated on 2-D gels, and the protein spots were excised and analyzed by MALDI mass spectrometry. Proteins that

were found in both the chaotrope and detergent-resistant fractions were considered as candidate NETs. In this way they could remove contamination from the ER and ONM proteins: as the chaotrope fraction contains both NE and ER transmembrane proteins, it alone is insufficient to distinguish INM proteins. This analysis identified most, but not all, previously characterized INM NETs, as well as mammalian Unc84A/SUN1 and a novel protein with no predicted functions that was named LUMA. Both novel mammalian NETs were shown to target to the NE by exogenous expression of the proteins fused to GFP [91].

Where the first study used a “comparative” approach to exclude peripheral ER proteins that also were present in the NE fraction, the second study from the Gerace and Yates laboratories used a “subtractive” approach [83]. In this case a microsomal membrane (MM) fraction was used to identify peripheral ER proteins. The MM fraction was analyzed separately from the NE fraction and all proteins appearing in both fractions were subtracted from the NE fraction. As there are no membranes in the nucleus besides the NE membrane and contaminating membranes of the NE fraction should in theory all also occur in the MM fraction, those transmembrane proteins in just the NE fractions were considered to be true NETs. Multiple NE fractions were also analyzed, but instead of using chaotropes an alkali extraction (0.1 N NaOH) was used to enrich for transmembrane proteins and salt and detergent (400 mM NaCl, 1% Triton X-100) were combined in one extraction to generate a cleaner lamina fraction. Combining both fractions resulted in a more comprehensive analysis of proteins with different biochemical characteristics as compared to the other study in which the different fractions were used to increase the stringency of inclusion. Fractions were analyzed using Multi-Dimensional Protein Identification Technology (MudPIT) [92,93], which couples tandem mass spectrometry with multiple liquid chromatography steps to analyze the complex mixture of peptides generated by direct digestion of isolated membranes. This avoids loss of membrane proteins that are poorly resolved on 2-D gels [94]. Details of the method are given in [95]. The subtractive approach was validated by the identification of all expected previously characterized NETs in the NE fraction and their absence from the MM fraction. Moreover, the numbers of peptides recovered suggests that

they are the most abundant NETs and this is why they were first identified by other means. In addition, 67 previously uncharacterized putative NETs were identified in the NE fraction, which were absent from the MM fraction [83]. All of the original eight tested targeted to the NE in the original study, suggesting that all would prove to be valid identifications; however of the 2/3 now tested only ~70% are valid NETs that are integral to the membrane and target to the NE (P. Malik, N. Korfali, N. Zuleger, V. Lazou, D. M. Kavanagh, G. S. Wilkie, D. G. Batrakou, and E. C. Schirmer, in preparation). Thus this method, like the NPC studies, still brings considerable contaminants. It appears, nonetheless, that some of this failure rate is not due to mis-identification or contamination, but rather to mis-prediction of transmembrane helices.

3.3 Comparison of benefits and disadvantages between the two approaches

The “subtractive” and “comparative” approaches used to identify NE-specific proteins both had limitations. The comparative approach disregarded NE-specific proteins that were not associated with the salt and nonionic detergent-insoluble lamina fraction, because no other basis was provided for distinguishing between the NE and ER transmembrane proteins that were present in the membrane-enriched fraction. The more conservative requirement of appearing in both membrane-enriched and lamina-enriched fractions also served to limit the number of identifications.

The subtractive approach had the disadvantage of disregarding proteins that have functions in both the ER and the NE. For example, a known ER protein, torsinA, appeared in both NE and MM fractions. However, torsinA is now known to move between the ER and INM where it interacts with multiple NETs [96].

While the subtraction limited the identifications, the combining all proteins in membrane-enriched and lamina-enriched datasets allowed for a more comprehensive analysis that covered a wider range of biochemical characteristics. This approach is not unreasonable considering the wide range of biochemical characteristics observed just for splice variants of one of the first identified NETs, LAP1 [87]. There were three variants of LAP1 recognized by a monoclonal antibody: the smallest was extracted with less than 200 mM salt, the intermediate sized variant was only about 50%

extracted at this salt concentration while the largest remained fully associated with the lamin polymer [15]. Non-equivalence has also been observed for different transmembrane proteins of the NE with regard to their extraction to chaotropes and different detergents. Just as empigen BB favored extraction of NPC components over other integral membrane proteins and lamins, partitioning of NETs in the first NE proteomic study indicated that emerin is more extractable by detergents than many other NETs while LBR is more extractable by chaotropes [91]. Thus the two studies highlight the persistent struggle between comprehensive identifications and contaminants.

Another reason for discrepancies between datasets is the randomness of getting single peptides into the mass spec and in obtaining good fragmentation to make identifications. In the present instance this was unlikely to be a primary contributor to variation as 92.7% of the proteins identified in the comparative study were also found in the subtractive study. Moreover 32.9% of the proteins identified in the NPC study were found in the comparative study (59.2% of core NPC proteins) and 89.3% in the subtractive study (if accepting variants or related proteins then this number jumps to 98.9%). This argues that the subtractive study was the most comprehensive, but the caveat as discussed above is that it likely also has the highest number of contaminants.

4. Recapitulation of other organellar functions in the nuclear envelope

4.1 NPC and NE proteins with dual roles

There are many previously characterized examples of proteins that have dual functions in the NE and other cellular structures or organelles. The first was Sec13p that functions in both ER vesicle formation [81] and NPC transport [82]. Additionally the DEAD-box helicases An3 and Dbp5 that bind RNA were also found to play an important role in RNA export [97-99]. Many core or peripheral NPC components have now been found to play separate roles on mitotic chromosomes when the NPCs are disassembled in mitosis. Ran, importin β and the Nup107-160 complex have been found to function in mitotic spindle assembly and on kinetochores [100-102].

NE proteins outside the NPC have also been found to have dual localizations and/ or functions. Lamins were originally thought to reside only under the inner nuclear membrane and provide structural support [60]; however in the past several years it has become apparent that lamins assemble and function also in the nucleoplasm [103] and on mitotic spindles [104]. Emerin is clearly an INM protein, binding to lamin A [14,18], the INM protein MAN1 [17], the chromatin binding protein BAF [105], the splicing factor YT521-B [106] and the transcriptional repressors germ cell-less, Btf, and Lmo7 [107-109], but emerin has recently been shown to function also in the outer nuclear membrane and ER [110]. Nesprins were named for their NE association, but are now recognized as a complex family containing many splice variants located throughout the cell [111]. Conversely Torsin A, like Sec13p, was originally characterized as an ER protein but is now known to normally sample the INM and accumulate there with certain point mutants associated with disease [112,113]. Torsin A has also been found to bind LAP1 in the INM and another protein that the authors renamed LULL1 and claimed resides in the ER [96]. However LULL1 was originally identified as NET9 [83] and has been confirmed in the INM [114]. Presumably NET9/LULL1, like Torsin A, can sample both compartments.

4.2 Newly identified NE proteins with apparent dual roles

Only a small percentage of the total proteins identified in the liver NE datasets were novel proteins with no predicted functions. Even among the 67 novel putative NETs identified, nearly a third had predicted functions based on sequence homology. An analysis of both predicted and characterized functional regions indicates a wide range of functions represented in both the NE and MM datasets (Fig. 2A; [115,116]). These functions range from transport and signaling functions to specialized functions of differentiated cells. Some of the proteins associated with cell signaling might converge on or add additional pathways to those recently identified at the NE [37-40]. A clear enrichment in proteins associated with DNA and RNA functions was noticeable in the NE compared to the MM. Lamins have been shown to have roles in DNA replication [41,42], transcription [45,46], and RNA splicing [47], but it is unclear whether lamin effects are direct or reflect their serving as

recruitment sites for specific enzymes. It has already been shown that transcriptional regulators pRb and germ cell-less respectively interact with lamins [46] and several NETs [17,44,109]. These proteins are among those identified in the liver NE dataset and so other proteins in this dataset that function in DNA and RNA processes might also mediate such lamin effects. Looking in more detail at the types of DNA functions represented in the NE dataset shows a striking enrichment of proteins involved in chromatin organization and remodeling compared to the total proteins in the human genome that function on DNA (Fig. 3A). For proteins functioning on RNA there is both an abundance and enrichment for those involved in RNA splicing and pre-mRNA processing (Fig. 3B). Though less abundant, there is also enrichment for those involved in mRNA end processing and stability, RNA localization and rRNA metabolism. This indicates that there are many potential mechanisms that could direct disease pathology from the NE involving disruption of gene expression beyond those currently being investigated.

Within the transporter category there were many pathways at play in addition to the expected NPC transport-associated proteins. This is not surprising in retrospect as regulated transport of smaller molecules such as ions should be important in the nucleus and potentially for disease, yet there has been very little focus in this area within the now chromatin-oriented NE field. Inositol(1,4,5) P_3 receptors were long ago reported at the NE [117,118], and specific ones were also found in the liver NE proteomic study that have been since directly tested [119]. Other regulators of Ca^{2+} transport and signaling were also found such as the ryanodine receptor, which has since been shown to function in the NE from several studies [120-122]. Indeed Ca^{2+} oscillations have been shown to affect gene expression [123] and so regulation of ion transport could also affect disease pathology through gene expression. Zn^{2+} transporters, Na^{2+}/H^{+} exchangers and many other ion transporters were also found in the NE proteomic datasets, many of which have since been directly shown at the NE [124,125], and there was a striking enrichment for electron transporters.

Detailed analysis of the enrichment in signaling proteins indicates that lipid signaling mechanisms in particular could be important in transducing signals to the nucleus in a separate or

backup mechanism to those that depend on transport of molecules through the NPC. In support of this NET39 that was identified in the liver NE proteomic study and confirmed at the NE [83] is a lipid phosphatase/lipid phosphotransferase of the candidate sphingomyelin synthase class and thus also now called CCS2 β [126]. This is the least studied subclass of the lipid phosphatase/ transferases, but others have been shown to play roles in regulation of cell growth and survival. There appear to be many proteins in the NE involved in these processes as NET13, also identified in the liver NE study, has now been shown to be a sphingomyelin synthase [127].

The various biochemical functions observed for these proteins and our confirmation of their partial representation in the NE has significant implications for many aspects of NE biology. Some studies have argued that nuclear membrane growth during interphase requires vesicle fusion or that ER membranes perfuse around the NPCs, but the identification of several enzymes involved in lipid generation in both NE datasets argues for de novo generation of lipid during interphase when the NE grows 3- to 4-fold. The recently reported function of NET8 (LPGAT1) as a phosphatidylglycerol acyltransferase [128] could serve to direct lipid content in the nuclear membrane, which has been reported to be one of the subcellular membranes containing phosphatidylglycerol [129]. The membrane trafficking function of NET24 (ERGIC-32; [130]) could have relevance for NE disassembly as Rab5 (which also appeared in the NE proteomics datasets) functions in NE disassembly in addition to its normal vesicle transport role [131]. Similarly, the signaling roles of emerin, MAN1 and AKAP149 [37-40] could be facilitated by functions of NETs 45 and 55 that respectively have been reported to directly function in signaling and to be a homolog of a protein involved in signaling [132-134]. Thus these NETs could be involved in signal transduction pathways similar to those of the well-characterized NET MAN1 in Smad/ BMP/ TGF β signaling [135].

5. Future Directions

5.1 Tissue variation in the NE proteome

There is precedent for tissue differences in NE proteins from the distribution of different lamin subtypes that have been shown to vary in relative concentrations during development and in different tissues [136-138]. There are also tissue-specific splice variants of lamins such as the lamin C2 that appears during spermatogenesis [139]. Although the first identified NETs were widely expressed, comparison of the novel NETs identified in the liver proteomic study to a transcriptome database [140] indicated that a significant percentage of the proteins recovered were preferentially expressed in liver and/ or had restricted tissue expression [13,141]. Moderate tissue variation has been reported recently in proteomes from mitochondria and other organelles [142,143]. A current study analyzing the NE proteome from several different tissues confirms that there is not only considerable tissue variation in the NE proteome but further suggests that there is more variation in the NE than these other subcellular organelles (N. Korfali, G.S Wilkie, E.A.L. Fairley, S.K. Swanson, D.G. Batrakou, P. Malik, A.R.W. Kerr, L. Florens, and E.C. Schirmer, in preparation).

The finding of tissue variation in the NE proteome together with observations that different epitopes on NE proteins are occupied in different tissues [144] argue that tissue-preferential binding partners of the NE proteins mutated in disease might mediate the tissue-preferential phenotypes of the wide range of NE-related diseases [13,145]. Different diseases preferentially affect muscle, neurons, bone, skin, heart, fat, and immune cells. Some diseases have partial overlap between a subset of these tissues. Thus a particular point mutant might disrupt a functional complex in one tissue where a specific partner within the complex is expressed, but have only a minimal effect on a different functional complex in another tissue where a different partner that has slightly different binding characteristics occupies the same general binding site. If this is the case, then determining the protein composition of the NE in different tissues may be critical to understanding these diseases. As variation will likely mostly occur in terms of relative amounts of protein as opposed to all or none effects, it will also be important for such future analyses to be performed using recently developed quantitative approaches such as SILAC, iTRAQ, and protein correlation profiling.

This also applies for the NPC as a study on the integral NPC component gp210 revealed that it has restricted tissue expression in mammals [146]. It is nonetheless essential for viability in *C. elegans* by RNAi [147]. Gp210 was also reported to be essential in HeLa cells [147] but other studies found that it could be deleted and that, rather, another integral NPC component, NDC1/NET3, is essential in HeLa cells [26,148,149]. Interestingly, they also found that NDC1 was not essential in *C. elegans* [148]. It is possible thus that different combinations of NPC components are expressed in different tissues, thus rendering them essential depending on the tissue being sampled. Accordingly it will be important to directly compare knockdown of several different NUPs over a panel of tissues. This, however, also means that aspects of nuclear pore structure and function may differ between different tissues and indicates a need to compare the NPC proteome in different tissues using identical extraction methodologies and quantitative approaches so that differences in extractability due to different detergents or salt concentrations used are not misread as tissue variation.

5.2 A further use of proteomics to gain structural insights into the NPC

The two NPC proteomic studies were considered at the time to be the definitive conclusion to NPC proteomics, but a recent yeast study indicates that mass spectrometry can contribute much more to understanding the NPC even without counting potential tissue variation. This study performed rapid pulldowns with each component of the NPC followed by quantitative mass spectrometry to identify binding partners and their ratios [30,150]. Stringent conditions were used so that by comparing the ratios of NUPs in different fractions those most likely to be in direct contact with one another could be ascertained. These data were combined with position measurements from immunoelectron microscopy for epitopes on NUPs and other available data to generate what is by far the highest resolution structure of the NPC to date [30]. This structure gives a more detailed view of the internal structure of the core NPC and the organization of the many core NUPs that is diagrammed schematically in figure 1C.

5.3 Additional NPC proteins in the NE proteome datasets?

It is noteworthy that the integral NPC component NDC1/NET3 was not identified in the mammalian NPC proteomic study [77], apparently due to its resilience to the extraction with empigen BB that was used to isolate the NPC proteins as their method relied on extracting NPC components from the membrane and lamina. NDC1/NET3 was, however, identified in the less conservative study of the NE that enriched for transmembrane proteins [83] and was later shown to be the third integral component of the NPC in mammalian cells [26,27,148] (it had previously been identified in yeast, but was too divergent for mammalian homologs to be identified by genome searching [23]), and this suggests that other proteins may have been missed in the NPC studies that appear in the NE proteomic datasets. If this is the case, it might account in part for the large differences between the masses predicted by electron microscopy studies and the final masses calculated from the proteins identified in the proteomic studies.

Searching the liver NE datasets for characteristics of NPC proteins such as the appearance of FG repeats indicates that proteins containing this motif are significantly enriched in the NE compared to the whole genome. FG repeats were observed in some of the first NUPs identified and it soon became apparent that they represented a common motif among most of the NUPs when three NUPs were identified using an antibody that recognized regions containing a GLFG motif: NUP49, NUP100 and NUP116 were found to have respectively 13, 29 and 33 GLFG repeats [151]. It is noteworthy, however, that only 10 of the 30 core NUPs have six or more FG repeats and 13 have one to five FGs (Table 1). Thus very large numbers may not be required for some FG functions. The FG repeat regions of NUPs are thought to be disordered [152] and tend to have >30% of polar residues between them. It has been suggested that the FGs within unstructured regions interact within the central channel of the NPC to provide an entropic barrier to passive diffusion [153], though the exact nature of that barrier is the subject of active debate [154,155]. One possible mechanism for transport through the entropic barrier would be exchange of FG interactions in the barrier with FG motifs on the surface of proteins associated with transport cargos [156]. The transport receptor importin β has 3 FG repeats so that coating the surface of the substrate cargo with importin β could potentially provide these FG

repeats on the outer surface to facilitate navigation through the central channel (Fig. 2B). If this hypothesis is correct, then other proteins that have FG repeats could similarly coat cargoes to facilitate their transport. A general search of all proteins in the human genome database reveals that only 1.86% have 5 or more FG repeats with 30% or more polar residues between at least 4 of them. In contrast, in the liver NE-enriched dataset 5.3% of proteins fulfill these criteria. It is particularly interesting that some DEAD box helicases have been shown to be important for nuclear export of mRNAs (including DEAD box protein 5 that has 5 FGs; [98,99]) and among the components of the TREX complex that is important for RNA export [157] is Sub2p, a DEAD box ATPase containing 2 FGs [158]. Strikingly, several of the liver NE proteins carrying multiple FGs are also RNA binding proteins including DEAD box proteins (Table 2). Thus if these stay bound to the mRNA during the process of transport they might facilitate the act of transport by coating the surface of the RNA with FGs (Fig. 2D).

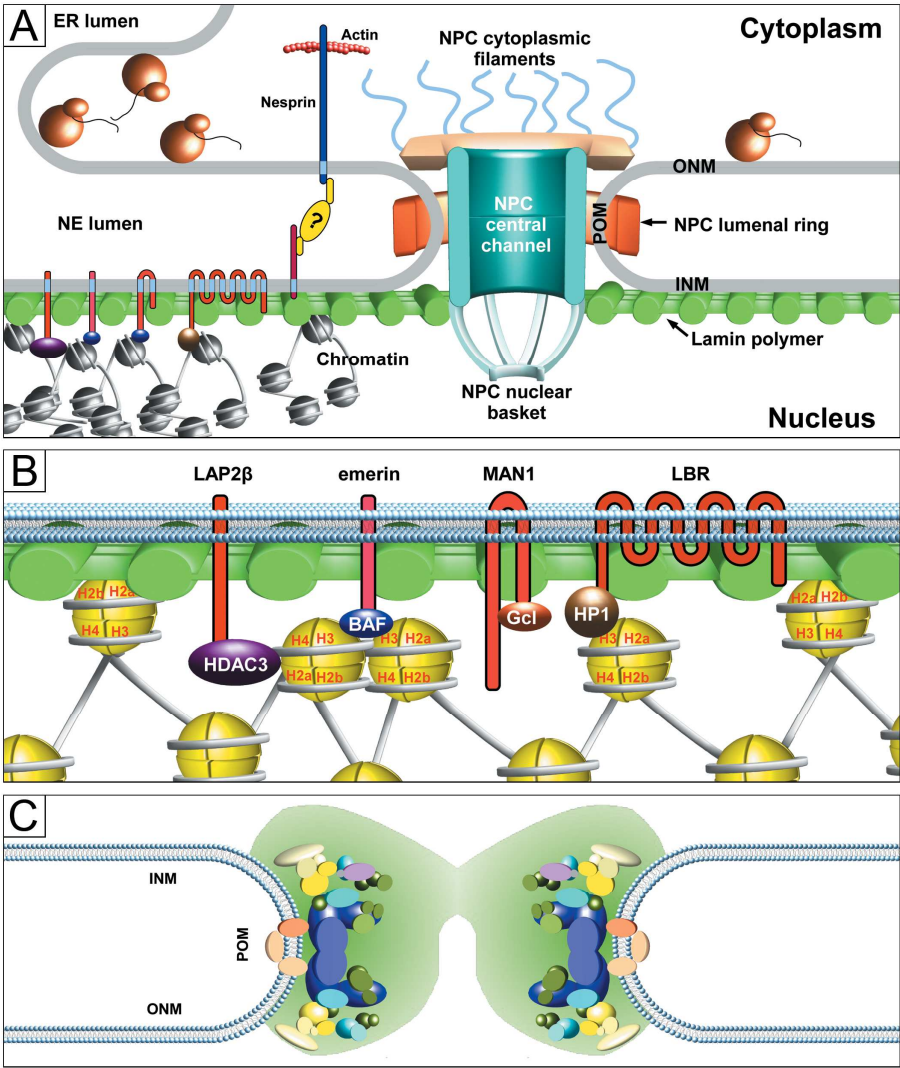
These observations indicate that the NE has a great many more functions and diversity than ever considered. To gain clarity of these functions it will be necessary to sample further methods of enrichment to reduce the number of contaminants and also to sample from different tissues, in both cases using new quantitative approaches. Even in the absence of further sampling, mining of the existing NE proteome datasets should yield considerable treasure for many years to come.

Acknowledgements

D.G. Batrakou is supported by the Darwin Trust: A.R.W. Kerr and E.C. Schirmer are supported by the Wellcome Trust.

Figure Legends and Tables

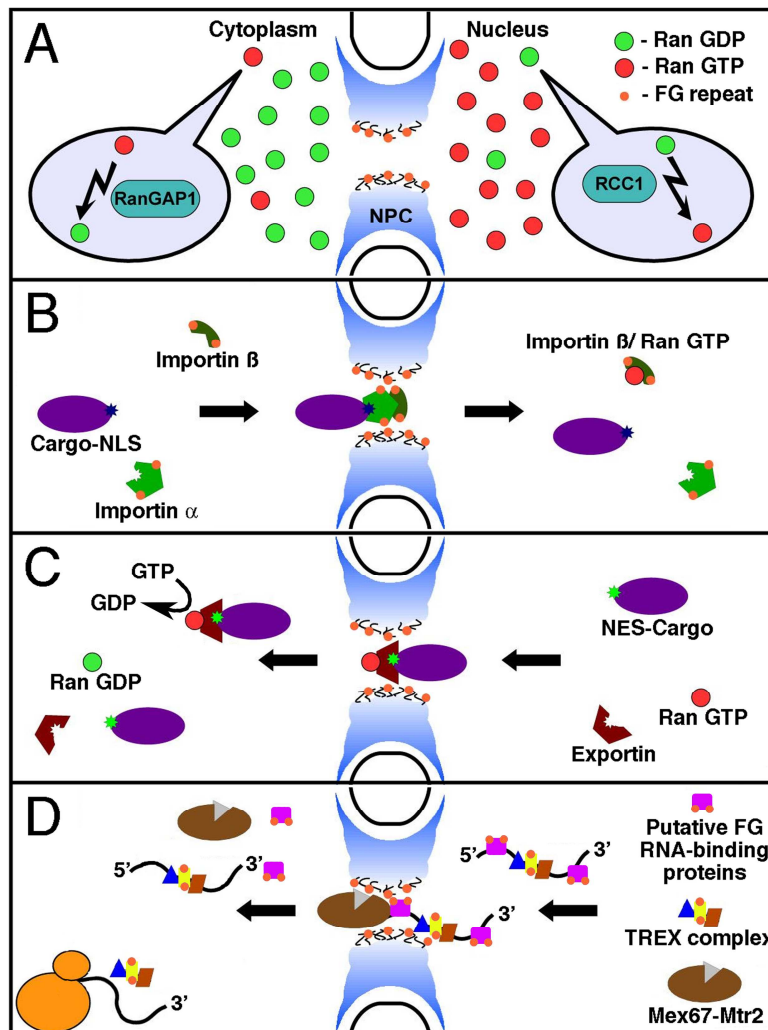
Figure 1.



Schematic diagram of the NE with details of the NPC. (A) The nuclear envelope consists of outer and inner nuclear membranes connected at the “pore membrane”. The outer nuclear membrane (ONM) is continuous with the rough and smooth endoplasmic reticulum (ER). The inner nuclear membrane (INM) contains many unique integral proteins, which commonly are associated with the intermediate filament lamin polymer. The pore membrane apposed to the nuclear pore complexes (NPCs) contains specific integral proteins involved in membrane tethering of NPCs. Depending on their topology and

membrane subdomain, NE transmembrane proteins could have functions in the cytoplasm, nucleoplasm, or the perinuclear luminal space. Some ONM-specific proteins have been identified such as the Syne/Nesprin protein families, but most proteins identified in this region such as ribosomal proteins share functions with the ER. Only a few characterized proteins have both been shown to contain most of their mass in the lumen, though more are likely based on proteomic results. (B) Many transmembrane proteins of the INM directly interact with the lamin polymer and/ or chromatin proteins, though only a small number of the first identified proteins have been tested for such characteristics. Among these, LAP2 β interacts with the chromatin remodeling protein histone deacetylase 3 (HDAC3). LAP2 β , emerin, and MAN1 all interact with the chromatin crosslinking protein BAF and transcriptional repressors such as germ cell-less (Gcl). LBR binds to both the heterochromatin protein HP1 and histone H3, and even lamins can directly bind to the core histones H2A and H2B. (C) The NPC proteomic studies identified many proteins that recently have been mapped into a high resolution structure (compared to electron microscope images; [30]). A depiction of this structure shows the rough positions of many nucleoporins and a density gradient of unstructured phenylalanine-glycine (FG) repeat containing regions (represented as a haze around the NPC core components) that are thought to play a significant role in the transport process.

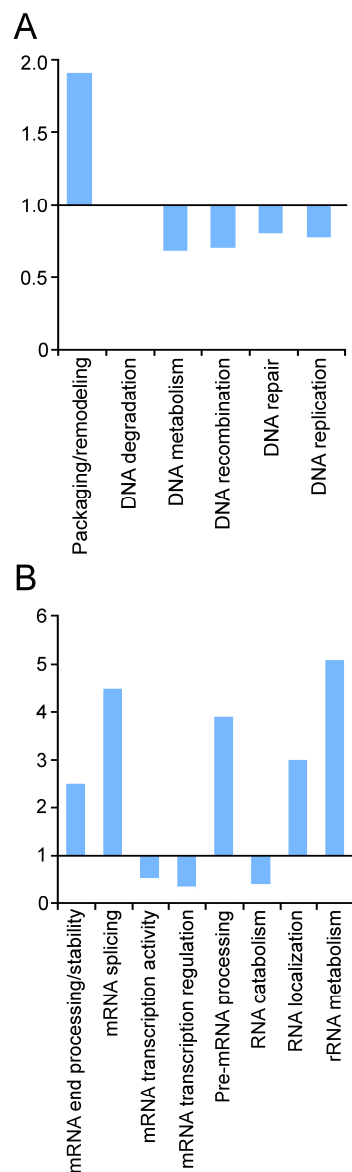
Figure 2.



Transport through the NPC. (A) Transport through the central channel of the NPC is facilitated by the action of the small GTPase Ran. RanGTP is concentrated in the nucleus and RanGDP in the cytoplasm. RanGAPs (Ran GTPase Activating Proteins) facilitate conversion of the GTP to the GDP form in the cytoplasm while RanGEFs (Guanine nucleotide Exchange Factors) such as RCC1 mediate replenishment of RanGTP in the nucleus. Ran is thus a very abundant protein that will be associated with NPC proteins even though it is not part of the core NPC structure. (B) In nuclear import a transport cargo that has a nuclear localization signal (NLS) (depicted as “*”) is recognized by the transport receptor importin α . Another transport receptor, importin β , then recognizes the importin α -

receptor cargo and facilitates its transport through the central channel of the NPC. This is thought to be mediated by interactions between the FG repeats on core NPC proteins and FG repeats on the transport receptors that now coat the cargo. Once the receptor-cargo complex has passed through the NPC RanGTP in the nucleus binds to importin β to facilitate dissociation of the receptor-cargo complex and thus release of the free cargo into the nucleoplasm. (C) In nuclear export a different kind of transport receptor called an exportin recognizes a nuclear export signal (NES) (depicted as “*”) on the cargo and binds together with RanGTP. The complex then transports through the central channel of the NPC and GTP hydrolysis initiated by the RanGAPs releases both the Ran and the exportin. (D) For RNA export several transport receptors have been identified such as the Mex67-Mtr2 heterodimer. Several additional proteins, some of which contain FGs, have also been found to be important that form a complex called the TREX complex in yeast. We propose that the many RNA-binding proteins that harbor FGs identified in NE proteomic datasets might be part of an even greater complex of RNA-binding proteins that coat the surface of the RNAs and expose FGs to interact with the FGs on the core NPC proteins and facilitate transport. The proteins shown in Table 2 are good candidates for this task.

Figure 3.



Functional distribution of proteins identified in the liver NE and MM proteomic study. (A) As subtraction of all proteins identified in the MM datasets would potentially miss interesting proteins that are nonetheless enriched in the NE, NE- and MM-enriched datasets were generated by comparing the number of spectra recovered per run for each dataset. If a NE protein had at least 5x more spectra than were recovered in the MM or vice-versa they were included in these datasets. The Panther (Protein ANalysis THrough Evolutionary Relationships) Classification System [115,116] was then

used to organize proteins in each dataset according to functional annotations. A clear enrichment for proteins involved in DNA and RNA functions (DNA/RNA) was observed in the NE compared to the MM. As only 2 proteins (<1%) recovered in the liver MM had anything to do with DNA function and 4 (1.7%) with RNA function, the % distribution of all DNA or RNA functions in the human genome was compared with that for the proteins recovered in the NE-enriched dataset. The ratio of the percent of a function in the NE to its percent represented in the total human proteome is presented. (A) DNA functions represented 65/854 proteins in the NE-enriched dataset compared to 977/25431 proteins in the total human dataset (or 7.6% vs 3.8%). Within the DNA set there was a strong enrichment at the NE for proteins involved in chromatin structure and remodeling. All other categories showed a relative decrease in functions at the NE. (B) RNA functions represented 330 of the 854 proteins in the NE-enriched dataset compared to 4295 of the 25431 proteins in the total human dataset (or 38.6% vs 16.8%). Within the RNA dataset functions in RNA splicing and pre-mRNA processing were both abundant and enriched for. Additionally functions in mRNA end processing and stability, RNA localization and rRNA metabolism were enriched for, though much less abundant.

Table 1. NPC core proteins [74,77] with number of phenylalanine-glycine (FG) repeats.

| Yeast | FG | Year (PubMed ID) | Mammalian | FG | Year (PubMed ID) |
|--------------|-----------|-------------------------|------------------|-----------|-------------------------|
| Nup1 | 17 | 1990 (2190694) | Nup153 | 30 | 1993 (8422679) |
| Nsp1 | 33 | 1990 (2112428) | Nup62 | 6 | 1990 (2295087) |
| Nic96 | 1 | 1993 (7688296) | Nup93 | 3 | 1997 (9348540) |
| Nup145N | 14 | 1994 (8044840) | Nup98 | 40 | 1995 (7878057) |
| Nup145C | 1 | 1994 (8044840) | Nup96 | 1 | 1999 (10087256) |
| Nup133 | 0 | 1994 (7813444) | Nup133 | 2 | 2001 (11564755) |
| Pom152 | 1 | 1994 (8138573) | — | - | - |
| Nup42 | 29 | 1995 (7634338) | NLP1/hCG1 (45) | 14 | 1999 (10358091) |
| Nup49 | 18 | 1995 (1385442) | Nup58 | 11 | 1991 (2050741) |
| Nup57 | 16 | 1995 (7828598) | Nup54 | 8 | 1991 (2050741) |
| Nup82 | 2 | 1995 (7559750) | Nup88 | 2 | 1997 (9049309) |
| Nup100 | 45 | 1995 (1385442) | Nup98 | 40 | 1995 (7878057) |
| Nup116 | 47 | 1995 (1385442) | Nup98 | 40 | 1995 (7878057) |
| Nup120 | 2 | 1995 (8557736) | Nup160 | 3 | 2001 (11684705) |
| Nup157 | 2 | 1995 (8522578) | Nup155 | 0 | 1993 (8458861) |
| Nup170 | 0 | 1995 (8522578) | Nup155 | 0 | 1993 (8458861) |
| Nup159 | 28 | 1996 (8898365) | Nup214/CAN | 45 | 1994 (8108440) |
| Nup188 | 5 | 1996 (8682854) | Nup188 | 3 | 2000 (11029043) |

| | | | | | |
|-----------|---|------------------------|-----------------|----|------------------------|
| Gle1(62) | 1 | 1996 (8848052) | hGle1(85) | 0 | 1998 (9618489) |
| Gle2(41) | 0 | 1996 (8970155) | Rae1/Gle2b (41) | 3 | 1997 (9370289) |
| Nup85 | 1 | 1996 (8816998) | Nup75/Nup85 | 2 | 1996 (8816998) |
| Nup84 | 0 | 1997 (9166401) | Nup107 | 3 | 1994 (8021268) |
| Ndc1 (74) | 3 | 1998 (9864355) | — | - | - |
| Nup53 | 4 | 1998 (9864357) | Nup35 | 3 | 2002 (12196509) |
| Nup59 | 6 | 1998 (9864357) | Nup35 | 3 | 2002 (12196509) |
| Nup192 | 4 | 1999 (10428845) | Nup205 | 4 | 2000 (11029043) |
| Seh1 (39) | 0 | 2000 (10684247) | Sec13-like | 0 | 2002 (12196509) |
| Nup60 | 0 | 2000 (10684247) | - | - | - |
| CDC31 | 0 | 2000 (10684247) | - | - | - |
| Pom34 | 1 | 2000 (10684247) | - | - | - |
| - | - | - | Pom121 | 24 | 1993 (8335683) |
| - | - | - | Gp210 | 5 | 1990 (2184032) |
| - | - | - | Nup358/RanBP2 | 20 | 1995 (7775481) |
| - | - | - | ALADIN (60) | 1 | 2004 (15666842) |
| - | - | - | Nup37 | 0 | 2002 (12196509) |
| - | - | - | Nup43 | 1 | 2002 (12196509) |
| - | - | - | Tpr (266) | 0 | 1994 (7798308) |
| - | - | - | Nup50 | 5 | 1997 (9073512) |

Table 2. RNA-binding proteins with FG repeats separated by polar residues found in the liver NE datasets.

| FG Repeats | Protein Name | Accession |
|------------|------------------------------------|----------------|
| 9 | hnRNP core protein A1 | NP_058944.1 |
| 9 | hnRNP A2/B1 isoform 1 | NP_058086.2 |
| 8 | RNA binding motif protein 19 | XP_222200.4 |
| 6 | hnRNP M | NP_446328.1 |
| 6 | hnRNP A0 | XP_001001311.1 |
| 6 | U5 snRNP-specific protein, 116 kDa | XP_001081526.1 |
| 6 | DEAD box polypeptide 42 | XP_001081592.1 |

Criteria used to search for FG repeat containing proteins were 5 or more FG repeats, at least 4 of which have $\geq 30\%$ polar residues between them (provided >3 residues separate the FGs).

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